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# Significant Long-Term, But Not Short-Term, Hippocampal-Dependent Memory Impairment in Adult Rats Exposed to Alcohol in Early Postnatal Life

***ABSTRACT:*** *In rodents, ethanol exposure in early postnatal life is known to induce structural and functional impairments throughout the brain, including the hippocampus. Herein, rat pups were administered one of three ethanol doses over postnatal days (PD) 4–9, a period of brain development comparable to the third trimester of human pregnancy. As adults, control and ethanol rats were trained and tested in a variant of hippocampal-dependent one-trial context fear conditioning. In Experiment 1, subjects were placed into a novel context and presented with an immediate footshock (i.e., within ~8 sec). When re-exposed to the same context 24 hr later low levels of conditioned freezing were observed. Context pre-exposure 24 hr prior to the immediate shock reversed the deficit in sham-intubated and unintubated control rats, enhancing freezing behavior during the context retention test. Even with context pre-exposure, however, significant dose-dependent reductions in contextual freezing were seen in ethanol rats. In Experiment 2, the interval between context pre-exposure and the immediate shock was shortened to 2 hr, in addition to the standard 24 hr. Ethanol rats trained with the 2 hr, but not 24 hr, interval displayed retention test freezing levels roughly equal to controls. Results suggest the ethanol rats can encode a short-term context memory and associate it with the aversive footshock 2 hr later. In the 24 hr ethanol rats the short-term context memory is poorly transferred or consolidated into long-term memory, we propose, impeding the memory's subsequent retrieval and association with shock.* © *2014 Wiley Periodicals, Inc.* Dev Psychobiol 56: 1316–1326, 2014.

***Keywords:*** *fetal alcohol spectrum disorder; context pre-exposure facilitation effect; hippocampus; memory consolidation; NMDA receptor*

## INTRODUCTION

Fetal alcohol spectrum disorders (FASD) encompass a constellation of physical and cognitive abnormalities that result from prenatal alcohol exposure in humans (Chasnoff, Wells, Telford, Schmidt, & Messer, 2010; Jones, 2011; Mattson, Crocker, & Nguyen, 2011). Afflicted individuals may exhibit a variety of structural and functional neurological deficits, including impaired executive function, attention, and learning and memory (Mattson et al., 2011; Niccols, 2007). Alcohol's teratogenicity depends critically on both the developmental period of exposure and the peak blood alcohol concentration (BAC), with the latter being a good predictor of FASD symptom severity (West, Goodlett, Bonthius, Hamre, & Marcussen, 1990). In the current study, binge-like ethanol exposure was limited to postnatal days (PD) 4–9 in rat pups, a period of central nervous

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system (CNS) development comparable to the third trimester "brain growth spurt" in humans (Bayer, Altman, Russo, & Zhang, 1993). Binge-like exposure produces higher peak BACs than the same amount of alcohol administered chronically (Bonthius, Goodlett, & West, 1988), and seems to better mimic alcohol consumption patterns in pregnant women with a history of alcohol abuse (Stephens, 1985). The developing hippocampus is vulnerable to ethanol during the postnatal period as demonstrated by reduced cell numbers, decreased morphological plasticity, impaired neurogenesis, and altered synaptic plasticity (Bonthius & West, 1991; Klintsova et al., 2007; Livy, Miller, Maier, & West, 2003; Puglia & Valenzuela, 2010). A number of hippocampal-dependent learning tasks have been used to investigate ethanol's deleterious effects across development—for example, both juvenile and adult ethanol rats are impaired in spatial and trace conditioning (Hunt, Jacobson, & Torok, 2009; Johnson & Goodlett, 2002; O'Leary-Moore, McMechan, Mathison, Berman, & Hannigan, 2006; Wagner & Hunt, 2006).

The acquisition and retrieval of context memories can also be manipulated in order to study hippocampal function (Fanselow, 2000; Rudy, 2009). A rat placed into a conditioning chamber and presented with an immediate footshock, for instance, shows almost no conditioned fear (as measured by freezing) to that context when subsequently re-exposed—a phenomenon termed the immediate shock deficit (ISD) (Bevins, McPhee, Rauhut, & Ayres, 1997; Fanselow, 1990). Subjects are unable to form a robust representation of the context in the limited time preceding the shock, restricting the context-shock association (Fanselow, 2000; Rudy, 2009). If pre-exposed to the conditioning context 24 hr prior to the immediate shock, however, subjects display significant conditioned fear when subsequently tested (Fanselow, 2000; Rudy, 2009), in what is known as the context pre-exposure facilitation effect (CPFE). Context pre-exposure is thought to promote binding of the various spatial and sensory features into a unitary or conjunctive memory, which can then be retrieved prior to, and associated with, the immediate shock (Fanselow, 2000; Rudy, 2009). The CPFE task is quite sensitive and able to detect even subtle deficits in context fear conditioning, making it an excellent tool for assessing hippocampal dysfunction (Rudy, Huff, & Matus-Amat, 2004; Rudy, Barrientos, & O'Reilly, 2002) including that linked to postnatal ethanol exposure in rats (Hamilton et al., 2011; Murawski & Stanton, 2010).

Beginning on ~PD70 the ISD and CPFE tasks were used to assess hippocampal function in control and ethanol-exposed rats. In Experiment 1, all groups demonstrated the ISD, but only ethanol rats were impaired in the CPFE task, displaying dose-dependent reductions in CPFE retention test freezing. The three CPFE sessions allow the acquisition (i.e., encoding and consolidation) of the context memory to be temporally isolated from its subsequent retrieval and association with the footshock. With that in mind, Experiment 2 was designed to test whether the ethanol-induced CPFE impairment is due to deficient encoding or consolidation of the context memory. Two time intervals (2 or 24 hr) were interposed between context pre-exposure and the immediate shock (PE/IS). If the context memory were properly encoded, we reasoned, a short-term memory would be available for association with the immediate shock when presented 2 hr later. In rats trained with the 24 hr PE/IS interval, however, context memory retrieval—just prior to the shock—requires the short-term memory first be consolidated into long-term memory, which depends on de novo protein synthesis for the maintenance of late-phase long-term potentiation (LTP) (Izquierdo & Medina, 1997). As such, a consolidation deficit was predicted if the ethanol rats displayed reduced freezing at 24 hr but normal freezing, relative to controls, at 2 hr—signifying the context memory was successfully encoded but inadequately consolidated into long-term memory.

## METHODS AND MATERIALS

### Experiment 1

***Subjects.*** Long-Evans male and female rats were housed in the vivarium at the University of Kansas. The animal colony room was maintained on a 12-hr light/dark cycle (lights on at 0700 hr) with ad-lib access to food and water. For breeding, one male and one female rat were housed together for 1 week. Beginning 3 weeks following their initial placement, female rats were checked morning and evening for parturition. Following birth, litters were culled to 10 pups, retaining equal numbers of males and females when possible. Rats were weaned on PD21, same sex housed through PD45, then individually housed through the end of testing. All procedures were in strict compliance with the University of Kansas animal care guidelines, and all necessary measures were taken to minimize pain and discomfort. A total of 75 rats from 15 litters were used in Experiment 1.

***Neonatal Treatment.*** On each day below rat pups were removed from the dam and placed in a small plastic container atop a heating pad and weighed. On PD3, the pups were injected with a non-toxic black ink into one or more paw for identification purposes. When possible,

one male and one female per litter were assigned to each of the five treatment groups: unintubated control (UC), sham-intubated (SI), or three ethanol groups, 3 g/kg/day (3E), 4 g/kg/day (4E), and 5 g/kg/day (5E). No more than one rat per sex per treatment group was used from any given litter. Over PD4-9 the ethanol pups were intragastrically intubated three times daily—two intubations with an ethanol/milk solution and a third milk-alone intubation—beginning at 0900 hr, with each intubation separated by 2 hr. The first two intubations consisted of 6.80% (3E), 9.06% (4E), or 11.33% (5E) ethanol in a nutritive milk formula (vol/vol), based on the recipe of West, Hamre, and Pierce (1984). PE10 tubing, lubricated with corn oil, was lowered down the esophagus into the stomach. The other end of the tube was connected to a 1 ml syringe containing the correct volume (.02778 ml/g of body weight) of the ethanol/milk or milk-alone solution. The SI pups were intubated three times daily, though no formula was ever given. The UC pups were removed from the dam three times daily but never intubated. Individual litters required approximately 15–20 min to complete, after which pups were returned to the dam.

***Blood Alcohol Concentration (BAC).*** Twenty microliters of blood was collected in heparinized capillary tubes from a tail clip in the SI and ethanol-exposed rats immediately before the final intubation on PD4. Blood samples for SI rats were discarded while samples from the 3E, 4E, and 5E rats were dispensed into microcentrifuge tubes, centrifuged, and plasma separated. An Analox GL5 Analyzer (Analox Instruments, Lunenberg, MA) was used to measure the rate of oxygen consumption resulting from oxidation of ethanol in the sample. Peak BAC was calculated by comparing an experimental sample to a known alcohol standard (100 or 300 mg/dl) used in the calibration procedure.

***Apparatus.*** Training and testing for all rats occurred in standard operant boxes (Coulbourn Instruments, Allentown, PA), contained within sound-attenuating chambers. Each operant box had two stainless steel walls, two Plexiglas walls, and a grid floor composed of .5 cm stainless steel bars placed approximately 1.5 cm apart. A small animal shock generator (model 82400; Lafayette Instruments, Lafayette, IN) and neon grid scrambler (model 58020; Lafayette Instruments) connected to the grid floor provided the footshock.

***Behavioral Procedures.*** Initially, UC rats were removed from their home cage and individually placed into 34 cm × 19 cm × 14.5 cm plastic cages cleaned with Windex®. Two cages were stacked, covered with a towel, and transported to the animal running room, a walk of approximately 5–6 min through public hallways. After 5 min rats were placed into the conditioning chamber and 8 sec later presented with a single footshock (2.0 sec, 1.5 mA), then removed 10 sec later. During the delay between transport and training, cages were placed beneath an unused workbench and covered with a sheet to minimize visual cues. Rats were transported in the same manner and with the same 5 min delay the following day before being placed into the conditioning chamber for 360 sec in order to assess contextual freezing. The ISD effect was not seen—that is, rats exhibited significant levels of freezing during the retention test—with a 5-min delay, presumably due to the duration and salience of the prior transport cues, which may be bound together with features of the physical context into a single conjunctive memory (Bevins, Rauhut, McPhee, & Ayres, 2000; Fanselow, 1990; Rudy et al., 2002). In order to facilitate the extinction of transport cues, and dissociate those cues from the conditioning chamber, further pilot work was done with delays of 60, 120, or 240 min between transport and placement into the chamber on both days. Freezing rates decreased with increasing delays, with the lowest rates of retention test freezing (i.e., the ISD) observed in the UC rats with a 240-min delay.

Across all treatment groups, half the subjects were trained in the ISD task; the remaining subjects were trained in the CPFE task. Two adult (∼PD70) rats were brought into the lab between 0900 and 1000 hr and the designated session began 240 min later, as described above. The inside of the running room was kept dark except for a single 40 W red light bulb. A fan inside each conditioning chamber (providing 60 dB background masking noise) was turned on and the inside was wiped down with a vinegar/water (1:5) solution immediately prior to the start of each session. For the context preexposure and retention test sessions, rats were placed into individual chambers and allowed to freely explore for 360 sec. For training, each rat was individually placed into their respective chamber and administered the footshock 8 sec later. They were removed after another 10 sec and returned to the vivarium.

***Freezing Analysis.*** Freezing was defined as cessation of all movement except that required for respiration (Blanchard & Blanchard, 1969). Freezing behavior was recorded with a black-and-white video camera (Model WDSR-2005SC; Circuit Specialists, Inc., Mesa, AZ) mounted to the top of the conditioning chamber. The interior of the chamber was illuminated by an infrared light source. The video signal was inputted to FreezeScan (CleverSys, Inc., Reston, VA), a video-based tool that can detect and quantify when subjects are motionless. Freezing was analyzed throughout the 360 sec pre-

exposure (CPFE) and retention test (CPFE and ISD) sessions.

## Experiment 2

***Subjects.*** Long-Evans male and female rats were housed and bred in the same manner described above in The Ohio State University vivarium. Subjects were maintained on a 12-hr light-dark cycle (lights on at 0600 hr), with ad-lib access to food and water. Rats were weaned on PD21, same-sex housed through PD60, then individually housed through the completion of the study. Body weight was not recorded in Experiment 1, which could have affected shock sensitivity and/or learning between treatment groups. Herein, rats were weighed across PD4-9, and again on PD10, 15, 21, 30, 45, and 60. All procedures were done in accordance with The Ohio State University Institutional Animal Care and Use Committee (IACUC), and all necessary measures were taken to minimize pain and discomfort. A total of 55 rats from 18 litters were used in Experiment 2.

***Neonatal Treatment and BAC Analysis.*** Rat pups from each litter were paw-marked on PD3 and assigned to one of three treatment groups: UC, SI, or 5E. The weighing, handling, intubation, tail clip, and BAC procedures across PD4-9 were identical to those described in Experiment 1.

***Apparatus.*** Each operant box was lit with a single 15 W bulb, located at the top of the sound attenuating chamber, and a pink geometric figure was attached to the front Plexiglas wall. The room outside the chambers was dark, lit only with a red overhead light. Speakers behind each chamber played ambient 60 dB white noise throughout all three sessions of the CPFE task.

***Behavioral Procedures.*** Experiment 1 established no treatment group differences in the ISD paradigm, whereas freezing in the CPFE test decreased with increasing concentrations of alcohol, consistent with other studies in ethanol-exposed rats (e.g., Murawski & Stanton, 2011). Thus, to minimize animal numbers just the CPFE task was used in Experiment 2, with the goal of determining whether the impairment seen in 5E rats was due to deficient encoding or consolidation of the initial context memory. Young adult (∼PD70) rats were pre-exposed to the conditioning context and then 2 or 24 hr later submitted to an immediate footshock (2 and 24 hr PE/IS interval groups). All groups experienced retention testing 24 hr after the immediate shock. Behavioral research space and the animal vivarium are adjacent at Ohio State and, consequently, the CPFE procedures were modified from Experiment 1.

Two rats were individually placed into 34 cm × 19 cm × 14.5 cm opaque black transport containers, cleaned with 5% ammonia, and carried to a quiet room adjacent to the training room. Rats spent 5 min in the transport containers while the conditioning chambers were cleaned with 5% ammonia. For pre-exposure and retention testing, rats were placed into the chamber and allowed to freely explore for 300 sec, after which they were returned to the vivarium. In the immediate shock phase, rats were individually placed into the chamber and after 5 sec administered a 1.0 sec, 1.0 mA footshock (model H13-15, Coulbourn Instruments, Whitehall, PA). Rats were removed from the chamber 8 sec later and returned to the vivarium. Freezing behavior was recorded and analyzed during the context pre-exposure and testing sessions with FreezeScan software, as described in Experiment 1.

Rearing behavior during the context pre-exposure session was quantified across the three treatment groups as a metric of context exploration. Active exploration (versus passive viewing) is necessary for rodents to form a contextual memory and benefit from context pre-exposure in the CPFE task (McHugh & Tonegawa, 2007). Rearing was defined as instances in which the rat stood on its hind legs with its forepaws above the original position of its head when on all-fours. Two forms of rearing were investigated: "on-wall" in which the rat touched a wall of the chamber with at least one paw and "off-wall" in which the rat did not touch a wall of the chamber. Videos were hand-scored by research assistants blind to treatment and PE/IS interval group.

## Data Analysis

BAC and behavioral data in Experiments 1 and 2 was analyzed using single-factor, multi-factorial, and repeated measures ANOVAs. Significant main effects or interactions were followed by Fisher's PLSD post hoc tests. A significant post hoc test implies $p < .05$. Due to low power, sex was not analyzed in the current study.

# RESULTS

## Experiment 1

***Blood Alcohol Concentration.*** Peak BACs are illustrated in Figure 1 for rats trained in the ISD or CPFE tasks. A 3 (Treatment) × 2 (Paradigm) ANOVA revealed a statistically significant Treatment group main effect, $F(2, 38) = 74.99$, $p < .0001$. Fisher's PLSD post hoc analyses revealed significant pair-wise contrasts across all comparisons, indicating the mean BAC for each ethanol treatment group was significantly different from

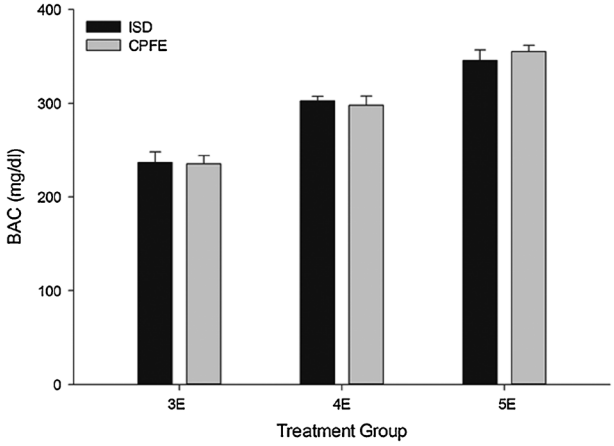


**FIGURE 1** Peak blood alcohol concentrations (BAC; mean ± SE) from Experiment 1, based on treatment group and task paradigm. Increasing concentrations of alcohol produced higher peak BACs. The mean BAC for each treatment group was significantly different from the remaining two treatment groups, though it did not differ between the two training tasks.

the remaining two. No differences were observed based on the training paradigm.

***Behavioral Findings.*** Five groups of rats, based on neonatal treatment, were trained with the ISD or CPFE tasks. Results are illustrated in Figure 2. Briefly, all groups demonstrated the ISD, with no significant differences between treatment groups. The CPFE on the other hand was significantly reduced in ethanol rats, with moderate (4E) and high (5E) dose rats impaired relative to controls.

***The Immediate Shock Deficit.*** Figure 2A illustrates averaged (±SE) freezing behavior across the 360 sec ISD retention test in UC ($n = 8$), SI ($n = 8$), 3E ($n = 8$), 4E ($n = 7$), and 5E ($n = 7$) rats. A single factor (Treatment) ANOVA revealed no significant differences between groups. The 6-min test was broken into 1-min bins and submitted to a 5 (Treatment) × 6 (Time) repeated measures ANOVA, revealing a significant main effect for Time only, $F(5, 165) = 9.11$, $p < .001$ (Fig. 2B), with freezing decreasing across the 6-min test. The results replicate previous findings indicating the immediate footshock was not successfully associated with the conditioning context in the absence of pre-exposure (Fanselow, 1990; Murawski & Stanton, 2011; Rudy et al., 2002).

***The Context Pre-Exposure Facilitation Effect.*** Figure 2A also shows averaged (±SE) freezing behavior across the 360 sec context pre-exposure and retention test sessions in UC ($n = 8$), SI ($n = 7$), 3E ($n = 7$),

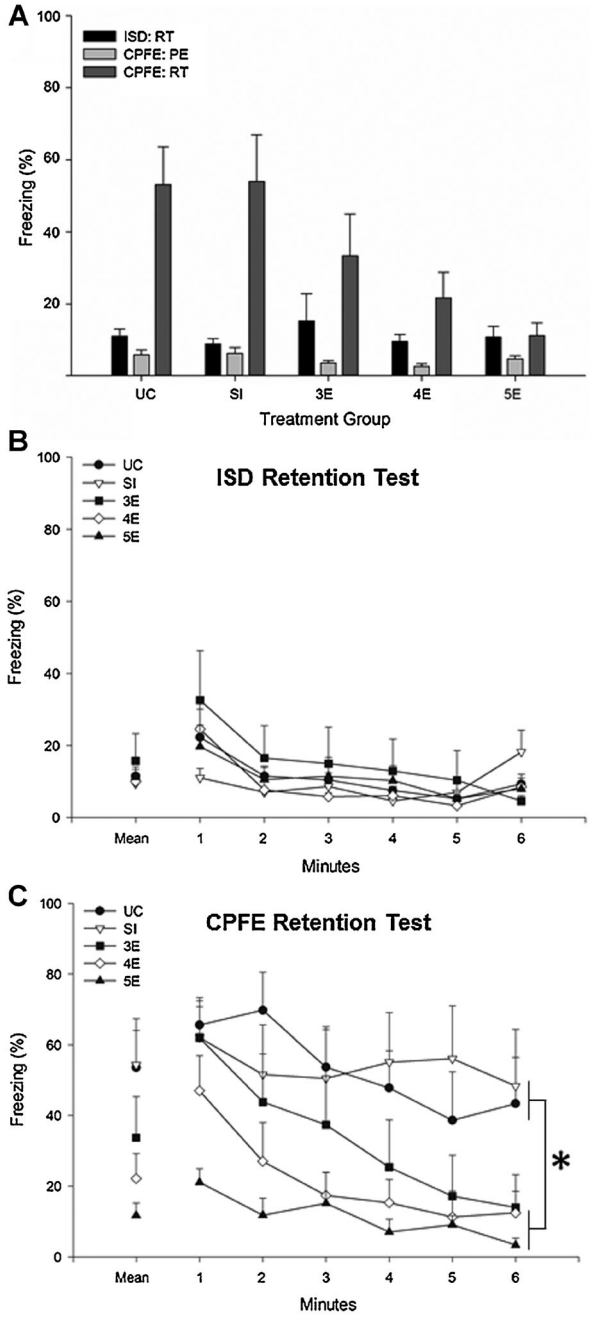


**FIGURE 2** Percent freezing (mean ± SE) across the ISD and CPFE tasks in Experiment 1. **A**: Averaged freezing rates across treatment group and training paradigm. For the ISD task, no treatment group differences in retention test freezing were seen (ISD: RT). For the CPFE task, neonatal treatment did not affect freezing during context pre-exposure (CPFE: PE). Retention test freezing was significantly affected (CPFE: RT), with the 4E and 5E rats freezing significantly less than control rats (not shown). **B**: Retention test freezing in the ISD rats based on 1 min bins. **C**: Retention test freezing in CPFE trained rats based on 1 min bins. The 4E and 5E rats froze significantly less than UC or SI rats, as indicated by the asterisk.

4E ($n = 8$), and 5E ($n = 7$) rats. Single factor (Treatment) ANOVAs applied to each session found no significant treatment group differences during context pre-exposure, whereas a significant main effect was seen for the retention test, $F(4, 32) = 3.80$, $p < .005$. Post hoc testing indicates the 4E and 5E rats froze significantly less than the UC and SI rats. Repeated measures ANOVAs, based on 1 min bins, again found no statistically significant main effects or interaction during context pre-exposure (data not shown), whereas retention test freezing was significantly influenced by Treatment, $F(4, 160) = 3.80$, $p < .001$, Time, $F(5, 160) = 17.86$, $p < .0001$, and their interaction, $F(20, 160) = 2.00$, $p < .01$ (Fig. 2C). Post hoc testing showed significantly reduced freezing in the 4E and 5E rats relative to the UC and SI rats. The latter group is the critical comparison, allowing for control of the intubation procedure. The significant interaction suggests the control rats displayed larger reductions in freezing over time than the ethanol rats, which were already freezing at low rates.

## Experiment 2

***Blood Alcohol Concentration and Body Weight.*** The mean (±SE) peak BAC in the 5E rats was 351 ± 14 mg/dl, similar to that seen in 5E rats from Experiment 1. Body weights (see Table 1) were recorded on select days across PD4 to PD60 in all rats. Treatment group effects were examined across the intubation period (PD4-9) via 3 (Treatment) × 6 (postnatal day) repeated measures ANOVA. Results revealed a significant treatment × day interaction, $F(10, 240) = 17.09$, $p < .0001$. Bonferroni-corrected one-way ANOVAs were performed on body weight over the same period, requiring (at 6 contrasts) $p < .0083$ for significance (maintaining a family-wise $\alpha = .05$). Significant treatment group differences were established across PD5-9, with smaller 5E rats, compared to UC and SI rats, beginning one day after initial ethanol exposure (PD5) through the end of the procedure (PD9). Neonatal treatment group effects were also analyzed on select days across development (PD10, 15, 21, 30, 45, and 60) though, due to substantial heterogeneity of variance, each day was analyzed separately via one-way ANOVAs. Results showed significant treatment group main effects on PD10, 15, 21, and 30 ($p < .01$, all 4 days). Post hoc analyses indicate the 5E rats weighed significantly less than both SI and UC rats on PD10 and significantly less than SI (but not UC) rats on PD15, 21, and 30. The UC rats were also significantly smaller than SI rats on PD30 only. Thus, while the 5E rats were initially underweight relative to controls, treatment group differences were mitigated as subjects grew toward adulthood, eliminating one potential confound.

**Table 1. Experiment 2: Mean Body Weights (g ± SE) Over Development for Each Treatment Group, Collapsed Across the 2 and 24 hr PE/IS Interval**

| | PD4 | PD5* | PD6* | PD7* | PD8* | PD9* | PD10* | PD15+ | PD21+ | PD30+$ | PD45 | PD60 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UC ($n = 17$) | 9.7 ± 0.3 | 11.7 ± 0.4 | 13.0 ± 0.4 | 14.8 ± 0.5 | 16.5 ± 0.6 | 18.4 ± 0.5 | 20.3 ± 1.6 | 28.3 ± 1.0 | 47.0 ± 2.0 | 91.1 ± 2.6 | 187.0 ± 6.8 | 268.3 ± 15.3 |
| SI ($n = 17$) | 10.1 ± 0.2 | 11.9 ± 0.3 | 13.7 ± 0.3 | 15.4 ± 0.4 | 17.4 ± 0.5 | 19.2 ± 0.5 | 21.4 ± 0.6 | 30.7 ± 1.0 | 51.2 ± 1.6 | 101.6 ± 3.0 | 200.2 ± 8.1 | 279.8 ± 17.0 |
| 5E ($n = 17$) | 9.8 ± 0.2 | 10.3 ± 0.3 | 11.3 ± 0.4 | 12.4 ± 0.4 | 13.7 ± 0.5 | 15.4 ± 0.6 | 17.3 ± 0.6 | 25.8 ± 0.9 | 41.8 ± 1.7 | 85.4 ± 2.8 | 178.6 ± 8.2 | 257.4 ± 16.1 |

Significant differences in body weights were noted between control (UC and SI) and 5E rats across PD5 to PD10, as indicated by an asterisk. The 5E rats were also significantly smaller than SI (but not UC) rats on PD15, 21, and 30, as denoted by the plus sign. Relative to UC rats, the SI rats were also significantly larger on PD30 (dollar sign). The "*n*" refers to data points, not subjects. See text for details.

***Behavioral Findings.*** Behavioral data from Experiment 2 is illustrated in Figures 3 (freezing) and 4 (rearing). Three treatment groups were submitted to the CPFE task, with context pre-exposure and the immediate shock separated by 2 or 24 hr. Similar rates of rearing behavior were seen across all groups during context pre-exposure. The 5E rats displayed significant reductions in retention test freezing following training with the 24 hr, but not 2 hr, PE/IS interval.

***The Context Pre-Exposure Facilitation Effect.*** Freezing behavior was recorded across the 300 sec context pre-exposure and retention test sessions in UC, SI, and 5E rats trained with the 2 hr ($n = 8$ per group; $N = 24$) or 24 hr ($n = 9$ per group; $N = 27$) PE/IS interval. In this case, "*N*" refers to the number of data points under analysis. There were four instances in which same-sex littermates were assigned to the same neonatal treatment × PE/IS interval group—their BAC and behavioral data were therefore averaged into a single data point (i.e., 55 rats were used for $N = 51$). Figure 3A illustrates averaged (±SE) freezing rates during the pre-exposure session in 2 and 24 hr rats. A 3 (Treatment) × 2 (PE/IS interval) ANOVA revealed no significant main effects or interaction. The same analysis applied to mean freezing during the retention test (left hand side of Fig. 3B,C) revealed a significant treatment group main effect only, $F(2, 45) = 4.09$, $p < .05$. Post hoc testing indicated the 5E rats froze significantly less than UC and SI rats when collapsed across both PE/IS intervals. Next, freezing behavior was analyzed within-subject across the 5 min retention test via 3 (Treatment) × 5 (Time) repeated measures ANOVAs at both PE/IS intervals. Neonatal treatment did not significantly influence freezing in the 2 hr rats (Fig. 3B), though there was a significant main effect for Time, $F(4, 84) = 20.38$, $p < .001$. In the 24 hr rats (Fig. 3C), significant main effects were observed for Treatment, $F(2, 96) = 5.21$, $p < .05$, and Time $F(4, 96) = 7.61$, $p < .001$, but not their interaction. Post hoc testing indicated the 5E rats froze significantly less than both control groups. Finally, we sought to determine whether retention test freezing within each treatment group varied based on the PE/IS interval. The 2 (PE/IS Interval) × 5 (Time) repeated measures ANOVAs revealed significantly less freezing in the 5E rats, but not UC or SI rats, when trained with the 24 hr versus 2 hr interval, $F(1, 15) = 5.43$, $p < .05$.

***Rearing.*** Rearing behavior was analyzed across the 300 sec context pre-exposure session (Fig. 4). No significant treatment group differences were noted in "off-wall" or "on-wall" rearing so the data was collapsed across rearing type. Results with a single-factor

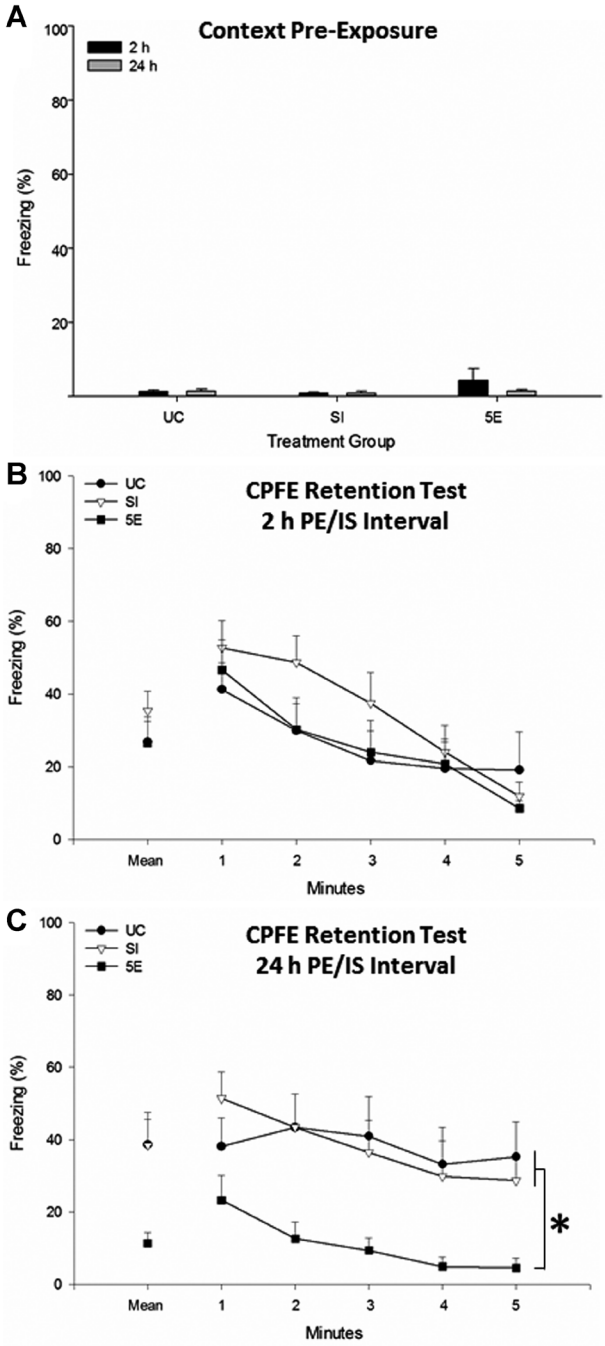


**FIGURE 3** Percent freezing (mean ± SE) across the CPFE task in Experiment 2. **A**: Averaged freezing rates across the pre-exposure session did not differ between treatment or PE/IS interval groups. **B**: Retention test freezing, across 1 min bins, with the 2 hr PE/IS interval. No significant differences were found between treatment groups. **C**: Retention test freezing, across 1 min bins, with the 24 hr PE/IS interval. The 5E rats froze significantly less than the SI and UC rats, as indicated by the asterisk.

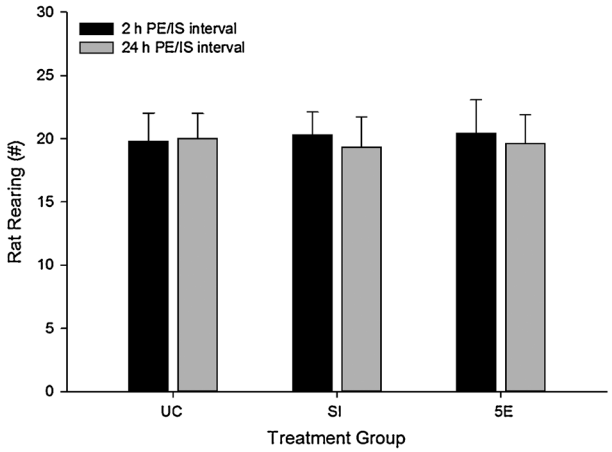


**FIGURE 4** Rearing behavior (mean ± SE) during the pre-exposure session in Experiment 2. No differences were found between treatment or PE/IS interval groups.

(Treatment) ANOVA revealed no statistically significant treatment group differences ($p = .80$), indicating roughly equal levels of activity and exploration across all groups of rats during the context pre-exposure session.

## DISCUSSION

In the present study, two variants of one-trial context fear conditioning were used to investigate the long-term neurodevelopmental consequences of binge-like, third-trimester equivalent ethanol exposure. Experiment 1 confirmed the ISD across all treatment groups, with minimal conditioning to the immediate shock in the absence of context pre-exposure (Fig. 2A,B). The behavioral results in CPFE trained ethanol rats showed a moderately linear decrease across dosage—that is, the 3E rats were unimpaired, relative to controls, while averaged freezing percentages were lower in 5E than 4E rats (Fig. 2A,C). Results roughly parallel those reported for both juvenile and adult rats following PD4-9 exposure (Hamilton et al., 2011; Murawski & Stanton, 2010, 2011), with significant reductions in retention test freezing observed in 4.0 and 5.25 g/kg/day ethanol rats (Murawski, Klintsova, & Stanton, 2012).

Third-trimester equivalent ethanol insult clearly induces long-lasting deficiencies in hippocampal function. The current results, together with other recent studies, show a relatively stable dose-response function from adolescence through adulthood in CPFE trained rats (Dokovna, Jablonski, & Stanton, 2013; Hamilton et al., 2011; Murawski & Stanton, 2010, 2011), indicating little recovery of function as animals mature. Standard context fear conditioning (i.e., placement followed by footshock 120 sec later), on the other hand, appears less sensitive to postnatal ethanol exposure. Relative to juvenile ethanol rats trained in the CPFE task, those trained with the delayed shock froze more during the context retention test but remained impaired relative to controls (Murawski & Stanton, 2010). Delay fear conditioning—but not trace fear conditioning (Hunt et al., 2009) — is even less sensitive to postnatal ethanol, with juvenile and adult ethanol rats acquiring and expressing CS-dependent fear at rates commensurate to controls (Schreiber & Hunt, 2013; Wagner & Hunt, 2006).

Notably, the current CPFE behavioral results show a high degree of generalization across Experiments 1 and 2, despite differences in lab location and conditioning procedures. In both experiments, rats trained with the standard 24 hr PE/IS interval (see Figs. 2C and 3C) displayed similar freezing rates during the first minute of the retention test, with elevated freezing in SI rats (62.1 ± 8.7% and 51.5 ± 7.3%) and diminished freezing in 5E rats (21.0 ± 3.9% and 23.2 ± 6.8%). Following training with the 2 hr interval (Fig. 3B) freezing rates in the 5E rats (46.6 ± 8.3%) were comparable to the UC rats (51.2 ± 7.4%) and SI rats (52.7 ± 7.4%). While postnatal ethanol exposure is known to induce hyperactivity in pre-weanling rats (Melcer, Gonzalez, Barron, & Riley, 1994), the 2 hr results clearly show adult 5E rats are capable of freezing behavior and the 24 hr CPFE impairment is not, therefore, attributable to a performance deficit.

In order to separate memory encoding and consolidation Experiment 2 varied the length of time between context pre-exposure and immediate shock, using a 2 hr interval in addition to the standard 24 hr (Fig. 3). With the latter, the context memory appears unavailable for association with the immediate shock in 5E rats, resulting in low levels of context freezing at test. This might occur because the context memory was only sparsely encoded, the memory trace dissipated over time due to poor consolidation, or the memory was stored but unsuccessfully retrieved. The 2 hr results, however, indicate the 5E rats froze at rates similar to UC and SI rats (Fig. 3B), suggesting the context memory was still sufficiently robust at 2 hr to be associated with the shock. Two additional points support our contention that the CPFE deficit in ethanol rats is not due to sparse memory encoding. First, Figure 4 shows nearly equal rearing rates—indicative of normal exploratory behavior—among UC, SI, and 5E rats during the pre-exposure session. Second, Dokovna et al. (2013) extended the pre-exposure period (5 min exposure followed by five additional 1 min exposures), resulting in only modest increases in freezing behavior during the CPFE retention test in SI juvenile rats. The ethanol rats (5.25 g/kg/day across

PD7-9) remained significantly impaired, however, suggesting the encoded context memory at the end of the initial 5 min exposure benefited little from the additional exploration time.

That said, the context memory formed during preexposure is presumably weaker than the subsequent context-shock memory, whose consolidation is aided by amygdala-dependent modulation in response to the aversive footshock (Huff & Rudy, 2004). While the current results cannot preclude a retrieval impairment in 5E rats for the context memory prior to immediate shock, they are clearly capable of retrieving a consolidated context-shock fear memory, as evidenced by the enhanced context freezing at test in 2 hr rats (Fig. 3B). In both cases, retrieval requires the context memory be formed and stored as a conjunctive representation via the co-active strengthening of N-methyl-d-aspartate receptor (NMDAR)-dependent connections within the CA3 recurrent collateral network (Kesner, 2007; Rolls, 2010). Once consolidated, even partial activation of the neural ensemble by the rat sampling environmental features in the brief time preceding the "immediate" footshock, for instance, should be sufficient to reactivate (or pattern complete) the entire conjunctive memory (Nakazawa et al., 2002). Thus, if the context memory were properly consolidated in the 5E rats then it should be retrievable prior to the immediate shock, as seen in controls. Alternatively, we suggest that the CPFE deficit may be linked to ethanol-induced alterations in NMDAR subunit composition and function (Hughes, Kim, Randall, & Leslie, 1998; Nixon, Hughes, Amsel, & Leslie, 2004; Samudio-Ruiz, Allan, Sheema, & Caldwell, 2010), which could, in principle, elevate the LTP induction threshold (Shouval, Bear, & Cooper, 2002) and limit or alter the downstream molecular cascades responsible for the short-term memory's consolidation (Samudio-Ruiz, Allan, Valenzuela, Perrone-Bizzozero, & Caldwell, 2009).

In summary, current findings indicate young adult rats exposed to ethanol during early brain development are dose-dependently impaired in the CPFE task using the standard 24 hr PE/IS interval. When shortened to 2 hr, however, the CPFE remained intact, signifying a robust hippocampal-dependent context memory can be encoded and associated with the footshock in 5E rats. The ethanol-induced learning impairment in 24 hr rats is proposed to lie, at least in part, in the long-term NMDAR-dependent consolidation of the labile short-term memory. Current results are expected to aid in our understanding of the long-lasting deleterious effects of perinatal alcohol exposure and, more broadly, which stage of acquisition during hippocampal-dependent Pavlovian conditioning might be particularly susceptible to disruption in FASD rodent models. Considering the well-documented forebrain-dependent learning and memory deficits seen over development in individuals with FASD (Coles, Lynch, Kable, Johnson, & Goldstein, 2010; Mattson, Riley, Delis, Stern, & Jones, 1996; O'Hare et al., 2009; Uecker & Nadel, 1998), future studies are needed to better define the specific mechanisms at fault within the hippocampus, including putative alterations in NMDAR structure, function, and downstream signaling.

## NOTES

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